SONY ID7000 protocols

(Manual written for ID7000 Software version 2.0.2)

Starting up the ID7000

1. Fill the sheath tank with autoclaved MiliQ water and empty the waste tank into the sink. Rinse the sink. (Note: consult local guidelines for proper waste disposal).
2. Switch on the two main power buttons on the left side of ID7000.
3. Press the power button on the front of the instrument.
4. Turn on the screen. (if the computer has been turned off, log in using workstation credentials).
5. Open the **ID7000 Software** (<u>not</u> *ID7000 Software Analysis*) and sign in to your user interface.
6. Wait for a popup asking for *Priming*, click *Start.* It takes 8 minutes (you can begin the protocol "Setting up an experiment on ID7000" while priming and running *Daily QC* in step 9).
7. Make AlignCheck beads in a 5 ml tube. Shake AlignCheck vial thoroughly, add 450 uL PBS + 2 drops of AlignCheck Beads to the 5 ml tube. Mix tube thoroughly before loading it into the ID7000. Align Check beads are valid for 3 days.
8. When Priming is done click *OK → OK.*
9. Click on *Start Daily QC →* Follow the wizard. It takes approximately 8 minutes.
   If the *Daily QC* fails, see below.
10. If QC passes, Click *OK → OK.*

If the sheath level becomes too low during a run the software will tell you. Go to Cytometer → Refill and empty → Follow the wizard. Once the wizard is done Go to Cytometer → Priming, click Start (takes 8 minutes).

If *Daily QC* (Align Check) fails:
1. Make new AlignCheck beads in PBS and rerun.
2. If it still fails, contact the FACS Core staff.

General note:

Every one to two weeks a pop-up window requiring *Optics Alignment* will appear. Make sure flow rate has been calibrated before following the wizard. Flow rate calibration is part of the daily QC, but if Daily QC has not been performed go to *Cytometer → Flow Rate*.

Experiment setup and running samples in Standardized Mode

Creating your color panel (Make sure your User Profile is set to Standardized Mode):

1. Experiment → *Experiment Designer* → Check lasers **On** in the bottom on the screen.

   If you are reusing a former experiment, go to **Reusing a former experiment as a template,** further down in this document.

2. To manually add fluorochromes to your panel, enter the name in the box at the bottom of the screen *Add New Spectral Reference* → Choose from the list if available → *Add* → Repeat for each fluorochrome.
   If the fluorochrome is not in the software, check **Nice to know – ID7000** on how to add it.
3. Enter the marker and other relevant information in the text boxes.
4. Click *Next*.
5. Choose rack or plate type. An Unstained sample is automatically added to A01.
6. If you do not use a common unstained control, then uncheck this box.
7. You can rearrange your single stain samples here.
8. Click *Next*.
9. For "Standardized Mode" → Click *Import* → *Browse* → Choose proper instrument settings for your ID7000 system → *Open* → *Import* → *OK* → Enable Low dead volume mode if you wish **(Nice to know – ID7000)** → *Next*
10. *Next* → Rename your experiment Name and Sample Group Information → *Next* → *Create Experiment*.
11. If you are missing any fluorochromes, go to Unmixing → *Unmixing settings* 🖼️ and add the fluorochromes you are missing.

Add and rename your samples:

12. To add an All Stain, FMO or other sample manually to your plate mark positions on the plate and right click → *Move to Sample Group.*
    Important: An unstained sample must be in the same sample group as your single stain controls.
    Remember to remove lids from your tubes/plate.
13. Mark the rack/plate under your experiment → List view ⊙≡ → Mark a sample and enter name.
    a. Or use the *Layout Editor* 🔲

Preparing your samples for preview:

14. Mark manually added samples → Right click → *Add to Auto Acquisition Target* (**Nice to know – ID7000**)
15. Click on 🎚️ Instrument →
    a. *Flow Control* → *Sample flow rate:* Keep it at 1.0 for the preview in step 18.
    b. *Agitation* → Choose your preferred settings.
16. Insert your plate/rack into the instrument.
17. First you must check if forward-, side scatter and threshold is appropriate. →
    Right click the unstained cell control (or any sample containing cells) in the plate view → *Set Current Position* → *Load Plate* 📤 → *Preview* ▶️

Open ⊪ Instrument →Detector and Threshold. Look at your cells in a forward- and side scatter plot and adjust FSC gain and/or SSC voltage if needed. Under "*Plot tools*" tab you can use "*Auto adjust (XY)/(X)/(Y)*" and "*Zoom*" features to adjust the scaling of your scatter plots.
Also adjust the threshold. An appropriate threshold will give you about 80-90% events inside your cell gate. Make a cell gate → Gate on singlets

18. Click on *Stopping Conditions* 🔴→ Choose singlets as your saturation gate → Choose your other stopping conditions and click *Sync Stopping Conditions* to add it to all samples in the sample group (**Nice to know – ID7000**)
Check that the stopping conditions have been transferred to the other samples.

19. Make sure your brightest all-stained sample does not go offscale → Right click the all stained sample you anticipate will be the brightest in the plate view → *Set Current Position* →*Preview* ▶ → If saturation rate in your singlet saturation gate exceeds 1% the signal is off scale, and PMT *voltages (%)* must be adjusted **(Nice to know - ID7000).** Remember to stop acquisition ■.

20. Also check that single stain controls are not off-scale → Right click the control in the plate view → *Set Current Position* → *Load Plate* 📤 → *Preview* ▶ → If the signal goes off scale or is too dim, you must prepare a new single stain control using a lower antibody concentration.

21. You can duplicate a plot and gate to all other samples in the sample group by right clicking a plot (not the gate) → *Sync Scale and Gate* → *Yes*

Acquisition of your samples:

22. Click on ⊪ Instrument → *Flow Control* → Adjust Flow Rate (look at the guide for approximate speed at different Sample Flow Rates) → *Auto Acquire* 🔴 . Remember to set auto acquisition preference to "Continue" after low event rate/air bubble encounters.

23. You can place gates around cells/beads of interest while the instrument is acquiring.

24. When acquisition is complete → Unload plate → Close experiment in Acquisition tab 📇 → Go to the protocol "Spectral Unmixing on ID7000"

Reusing a former Standardized Mode experiment as a template:

1. Experiment → *Experiment Template* → Mark the experiment of choice → *Create Experiment*
2. Mark a sample group → *Import Instrument Settings* → *Browse* → Choose proper instrument settings → *Import* → *OK* → *OK*
3. Repeat step 2 for each sample group!
4. Remember to change FSC gain, SSC voltage and threshold values specific to your experiment.

Reusing a former Standardized Mode experiment with data:

1. Experiment → *Reopen Experiment* → Mark the experiment of choice → *Reopen in Acquisition*
2. Mark a sample group → *Import Instrument Settings* → *Browse* → Choose proper instrument settings → *Import* → *OK* → *OK*
3. Repeat step 2 for each sample group!
4. Remember to change FSC gain, SSC voltage and threshold values specific to your experiment.

Spectral unmixing on ID7000

Open ID7000 ID_A (analysis) software

Log in.

Go to Analysis → Select the experiment you would like to open and click on it.

Select the sample group with data.

Choosing positive signals for single stain controls:

1. Go through your single stain controls → Adjust the positive gate in each of them. Make sure that you have a clean spectral pattern in your positive and negative ribbon plots (see image below). If you create new gates instead of using the automatically added Positive gates, right click the new gate → *Assign Gate* → *Positive gate* - Choose the correct parameter.

2. Go to your unstained sample. Make a FSC VS SSC plot and mark unstained beads/cells -> Right click gate and assign as universal negative control.

3. Unmixing → *Unmixing Settings.* Make sure positive and negative gates have been correctly assigned (If you have been utilizing different beads (or beads and cells) for single stains, remember to assign proper negative gates for each of them.

4. Press *Calculate* and look at spectrum for each fluorochrome. If any spectral line goes below zero you need to review the positive and/or negative gates of that fluorochrome. If any warning signs show up hover mouse over them to see details.
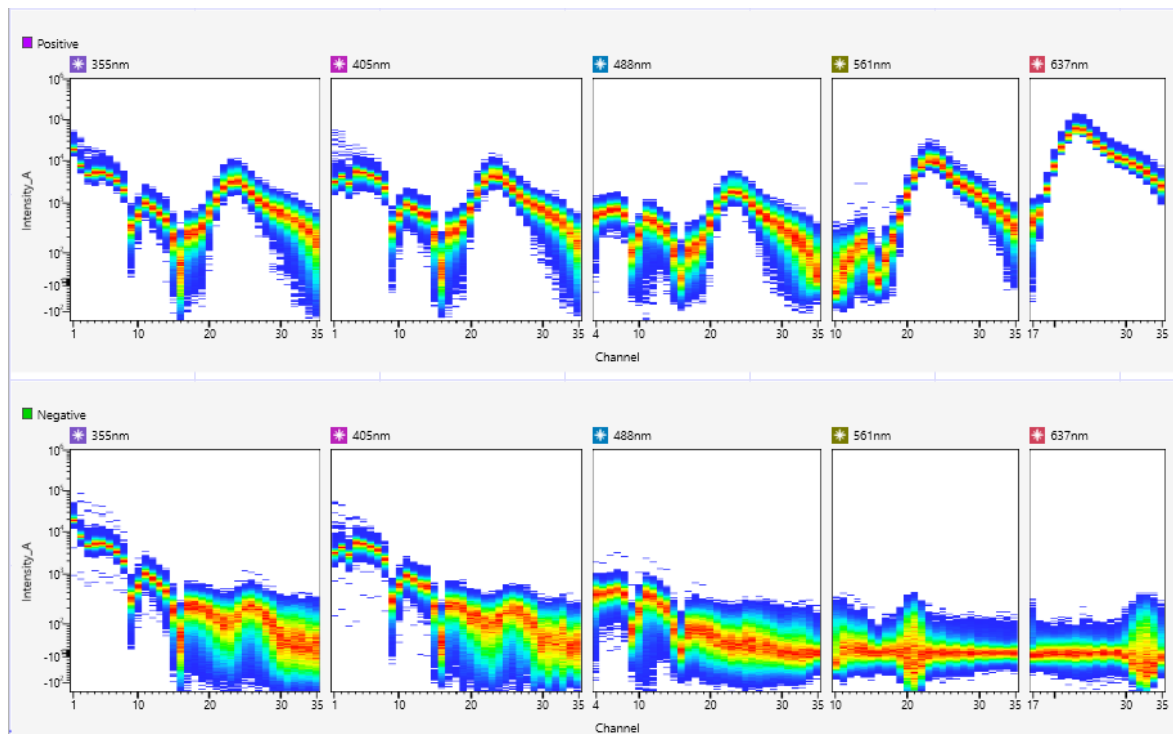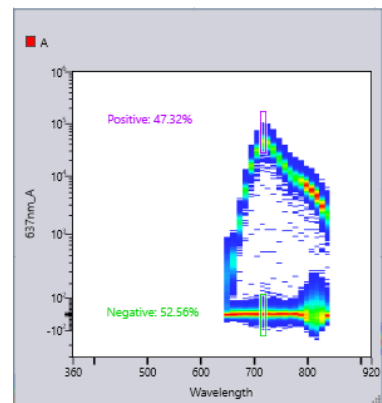
Determining autofluorescence in universal negative cell sample (ID7000 autofluorescence SOP):

5. Ensure your unstained sample is in the same sample group as your single stained samples. If not → right-click your unstained sample → move to sample group → single stained group.

6. Mark your unstained sample → Unmixing → *Autofluorescence Finder* → OK

7. *Display Event* → Choose the number of events to be displayed (we recommend 50,000)

8. Step 1: Place a gate around your cells of interest in the FSC-SSC plot.

9. Step 2: Change the Y-axis to *[VF-355]* → Use the sliders below the plots to increase separation of populations → Highlight the plot you want to make a gate in → Place gates around the different autofluorescent cells → *OK* → *OK* → Check "Calculate without saturated events"

10. Go to Unmixing → *Unmixing settings.* Check that spectrum for the autofluorescent populations are not below zero. Chose *Calculate* → *Apply.* Now the unmixing is calculated.

Review unmixed all stain sample:

11. Ensure your all-stained cell sample is in the same sample group as your single stained samples. If not → right-click your unstained sample → move to sample group → single stained group.

12. Mark your all stain sample → Place relevant gates to remove debris and doublets → Remove dead cells before you continue.

13. Unmixing → *Unmixing Viewer* → Check the On → Mark all your plots (you can use Shift) → *Auto Adjust XY* to adjust the axis.

14. You may need to adjust the axis further e.g the linear area around zero.

15. Skim your plots for "banana shapes" in the positive populations (For an example of a "banana shape", see next page). If all looks good → *Next* in the upper left corner.

16. Any plot(s) to the left of the perfectly diagonal plot have already been reviewed by you.

17. Find the plot where you see the worst unmixing issues (most severe "banana shapes").

Manually adjusting of unmixing:

18. Unmixing → *Spectral Reference Adjuster*

19. Start with the worst plot → Adjust the unmixing by dragging until the banana shapes are gone (figure 1). Adjusting the worst plot can sometimes fix issues in other plots.

    Important notes:
    Take biology into consideration in this step.
    Double check fluorochrome assignment if unmixing looks bad.
    Some fluorochromes perform differently on beads and cells.
    If you have many issues, your panel might need to be redesigned.

20. Repeat step 18 until you have reviewed all fluorochromes.
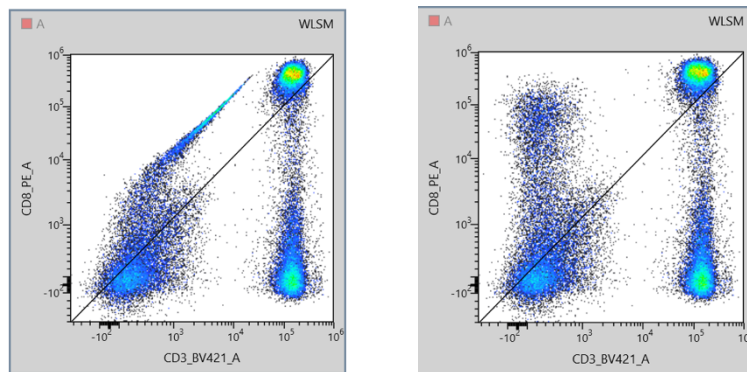
Figure 1: "Banana-shape" to the left and correct unmixed populations to the right

21. When you are done, click *OK* → Name the unmixing matrix as you prefer → *Save.*
22. Save whenever you have made larger adjustments.
23. Verify your unmixing. Go through all plots again (see review unmixed all stain sample).
24. Close.

Last steps:
25. Acquisition → *Close* → You are automatically moved to Analysis. You might want to verify your unmixing in 2D plots – check your gating strategy. Right click your experiment → *Export to FCS file* → Chose the correct unmixing matrix→ Make sure the right path is chosen → *Export* → *OK*

When you are done using the ID7000

1. *Unload* 🗔
2. *Close* 🖳
3. If you have been running samples from **solid tissue** (splenocytes and lymph node cells are exempt) go to **step 4**. If not, go to step 10.

4. Experiment →*Reopen Experiment → Public → Chose "NovoRinse Cleaning" (for tubes).* Say *"Reopen in Acquisition".*

5. In the refrigerator a tube with NovoRinse (soap) can be found. Load Plate 🗔. Set current position to the place you have the tube with NovoRinse. Click 🎚️ Instrument
   a. Flow control → Set Flow rate at 10
   b. Event check → Set to off
6. Run for 1 min in preview.
7. *Unload Plate* 🗔
8. *Close*
9. Go to Cytometer → *Flow Cell Purge* (takes approx. 5 minutes).

10. Cytometer → *Decontamination* ✨ → *Bleach Cleaning and Rinse* → Follow the wizard (takes approx. 10 minutes)
    Both cleaning reagents can be found in the refrigerator.

    Remember to write the date you open a new H$_2$O tube or make a new 1:5 chlorine dilution. They last 7 days.

    a. *Rinse in this wizard is H$_2$O from the 5 ml tubes in the back of the refrigerator.*
    b. *Bleach is diluted 1:5 Fx.: 750 µl Bleach from the glass bottle and 3000 µl H$_2$O.*

11. See if someone has booked the instrument after you.
    If there is a user after you go to step 12 under **User change** below.
    If not, go to **Shutting down ID7000** on the next page (step 21).

User Change

12. Call the next user. Their mobile number can be seen by placing the cursor over the next user's reservation in the iLab calendar.
13. If you cannot get in contact with the next user you have to contact the FACS Core Facility personnel.
14. Outside normal work hours, close the instrument if you cannot reach the next user.
15. Sign in to your server using the protocol "Connect and save data to network share".
16. Export your experiment file and/or FCS files.
    a. Experiment file: File → *Database → Export* → Choose your experiment(s) → Move → Browse → Choose a folder on your server → *Export*.

b. FCS files: Analysis → Check the experiment(s) you want to export → Right click → *Export to FCS file* → Here you can choose to check/uncheck what to export. To export with an unmixing matrix, make sure to click on a sample group and choose the matrix → *Browse* → Choose a folder on your server → *Export*.

17. You may have a maximum of 5 experiments on this computer. Delete any excess experiments in Analysis.

18. Sign out if the ID7000 Software.

19. Go to Windows File Explorer → Right click your server → *Disconnect*

20. Remove the cleaning tubes and put back in the front rack in the refrigerator.

Shutting down ID7000

21. Sign in to your server using the protocol "Connect and save data to network share".

22. Export your experiment file and/or FCS files.
    a. Experiment file: File → *Database* → *Export* → Choose your experiment(s) → *Move* → Browse → Choose a folder on your server → *Export*.
    b. FCS files: Analysis → Check the experiment(s) you want to export → Right click → *Export to FCS file* → Here you can choose to check/uncheck what to export. To export with an unmixing matrix, make sure to click on a sample group and choose the matrix → *Browse* → Choose a folder on your server → *Export*.

23. You may have a maximum of 5 experiments on this computer. Delete any excess experiments in Analysis.

24. Close the software.

25. Turn off the two main power buttons on the left side of the instrument.

26. Remove the cleaning tubes and put back in front rack in the refrigerator.

Nice to know – ID7000

Tips for Setting up a new experiment

1. <u>Adding new fluorochromes</u>:

   First, make sure that the fluorochrome is not on the list. Try abbreviations or full name of the fluorochrome.

   If not on the list, then enter the name and click *Add* → Find a spectra viewer

   - **Name**: Check the spelling of the name.
   - **Laser**: Choose the correct excitation laser in the spectra viewer.
   - **Start channel:** Look in the spectra viewer and determine where the emission peak starts and choose a channel that corresponds well to the peak.
   - **End channel:** Again, look in the spectra viewer and determine where the emission peak ends and choose a channel that corresponds to the end of the peak.
   - **Color:** Choose a color to display in the software.
   - **Spectral:** If you know that the fluorochrome has the same emission spectrum as another fluorochrome, you can choose it from the list, if it is there. If not, you can leave this blank. It does not have a relevant function in your unmixing process, it will just show the emission spectrum in the diagram below.

   It is possible to not choose anything else than the name and leave everything else as default but choosing the correct settings will make it easier placing the correct gate in step 6-7 in "Spectral Unmixing on ID7000" protocol.

2. <u>Enable Low dead volume</u> This will add 10 seconds to each well. If you are using compensation beads from Invitrogen they sediment fast – here you should active low dead volume mode (avoid those if possible).

3. *Add to Auto Acquisition Target* Samples added to auto acquisition targets will all be acquired when using the *Auto Acquire* . You can recognize which samples are added to auto acquisition by the filled circle in the plate view vs .

4. <u>Sync stopping conditions</u> A sample group with a chain means that they share the same worksheet with plots and gates and share Stopping Conditions (all settings are the same). Single stain controls (or Single Positive Controls, as the software calls the sample group by default) will not have a shared worksheet.

   If you change your Single Positive Controls sample group to a shared worksheet, you will lose at the automatic positive gates in the ribbon plot then when getting to the gating steps in "Spectral Unmixing on ID7000" protocol.

   Therefore, it is recommended to use individual worksheets for Single Positive Controls.

General tips

5. Ctrl Z = undo and Ctrl Y = redo

6. <u>To compare ribbons</u>, right click the given ribbons and send to overlay

7. <u>Changing general preferences</u> for your user profile:

    Go to "File", chose "Information", then "Preference Settings". You can make the default settings as you wish. For example, under "Instrument settings", if you mainly work with MNC, you can change the FSC gain to 10.

8. <u>Change between normal or standardization mode</u>. Right click on the *Experiment folder* 🖽 → *Properties* – here you can switch from normal to standardized mode. Go to 🎚 Instrument → *Detector & Threshold*. In standard mode, set all PMT voltages to 5.

    - Keep the instrument settings window open.
    - Run as preview (sample speed slow; 1), set threshold and any sample you are afraid will go off scale (E.g. PE og PE-Dazzle594) check that.
    - After running, check each individual single stain. Gate on brightest peaks in the ribbon (and remove doublets, triplets) so you get a nice tight spectral ribbon.

Tips for unmixing

9. If you are using cells as single stains:

    - If you start with a lymphocyte gate as a negative control gate and afterwards look at e.g. a granulocyte marker, then move the negative gate to the granulocytes for this sample → Right click → Assign Gate → Negative → Choose the fluorochrome.

    - If a spectrum is messy you can set your negative gate for a given fluorochrome underneath the positive gate in the ribbon plot. This could help clean up the spectrum.

    - If you are looking at a rare marker, or if two single stains are mixed in one sample, then you can find each fluorochrome by gating on the most positive events in the ribbon plots → Show the spectrum on a ribbon plot → Place and adjust gates until you have removed noise and outliers.

10. <u>Unmixing errors typically arise from fixed versus unfixed cells. Bad/old lot of beads. Or a broken tandem.</u>

11. You can save the spectral signatures of your fluorochromes to ID7000 library. Then you can reuse them anytime you need them. Be cautious when reusing tandem dyes as these can decay over time and hence change their spectral signature.

12. You can also export the entire unmixing matrix and bring it with you to another ID7000 instrument. However, remember that you need to run your experiments in standardized mode on both instruments to be able to do this.

13. You can setup the instrument to automatically shutdown after running your samples. Just activate the automatic shutdown feature, load cleaning reagents into the washing station and press auto acquire. The ID7000 will run the cleaning procedure after your samples are done and subsequently shut down.